

Conspirators in a Capital Crime: Co-deletion of p18^{INK4c} and p16^{INK4a}/p14^{ARF}/p15^{INK4b} in Glioblastoma Multiforme

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Abstract

Glioblastoma multiforme (GBM) is one of the most dreaded cancer diagnoses due to its poor prognosis and the limited treatment options. Homozygous deletion of the p16^{INK4a}/p14^{ARF}/p15^{INK4b} locus is among the most common genetic alterations in GBM. Two recent studies have shown that deletion and mutation of another INK4 family member, p18^{INK4c}, also drives the pathogenesis of GBM. This minireview will discuss the known roles for p18^{INK4c} in the initiation and progression of cancer and suggest opportunities for future studies. [Cancer Res 2008;68(21):8657–60]

Introduction

Glioblastoma multiforme (GBM) is one of the most devastating human cancers, notorious for its fast growing nature, infiltrative growth, resistance to radiotherapy, and rapid progression from diagnosis to death (1). Homozygous deletion of the p16^{INK4a}/p14^{ARF}/p15^{INK4b} locus on chromosome 9p21.3 is a signature genetic event that drives the pathogenesis of GBM (2, 3). Deletion of this locus is thought to be strongly selected for during the pathogenesis of GBM and other tumor types because it simultaneously inactivates three tumor suppressor genes: the p16^{INK4a} and p15^{INK4b} cyclin-dependent kinase (cdk) inhibitors and p14^{ARF}, which modulates the activity of the p53 tumor suppressor (4, 5).

p16^{INK4a} is the prototype member of the INK4 family of cdk inhibitors, which includes p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}. These four proteins have ~30% amino acid sequence similarity and share potent cyclin-dependent kinase (cdk) inhibitory activity, but are thought to differ from each other in their patterns of expression and their relative affinities for different cdks. Of the four genes, p16^{INK4a} plays the most prominent role as a tumor suppressor gene in human cancer, as it is deleted or mutated in the majority of common human tumor types and the cause of an inherited cancer predisposition syndrome. In contrast, p15^{INK4b} and p18^{INK4c} are thought to play more limited roles as human tumor suppressors, and p19^{INK4d} is thought not to be involved in cancer pathogenesis.

Two recent studies have shown that deletions and mutations of p18^{INK4c}, together with deletions and mutations of the p16^{INK4a}/p14^{ARF}/p15^{INK4b} genes, drive the pathogenesis of human GBM (6, 7). When taken together with other prior studies identifying deletions and mutations of p18^{INK4c} in several other tumor types

and showing that p18^{INK4c}-deficient mice are tumor prone, these recent studies have suggested that inactivation of p18^{INK4c} may play a perhaps underappreciated role in human cancer pathogenesis. As such, this review will provide a brief history of the discovery of p18^{INK4c}, summarize the studies that link it to cancers in mice and humans, and provide a forward-looking assessment of the role of p18^{INK4c} inactivation in the pathogenesis of GBM (Fig. 1).

Discovery of p18^{INK4c}

The human p18^{INK4c} gene was initially discovered in 1994 by Guan and colleagues (8) in a yeast two-hybrid screen to identify proteins that interacted with human cdk6. The mouse homologue was reported the following year by Hirai and colleagues (9). Human p18^{INK4c} is a 168-amino-acid cdk-interacting protein with multiple ankyrin repeats and substantial similarity to both human Notch and the yeast cdk inhibitor PHO8. p18^{INK4c} bound with high affinity to cdk6, with intermediate affinity to cdk4, and did not bind to cdk2. This discovery of a new p16^{INK4a} homologue immediately raised the exciting possibility that p18^{INK4c} might be a new tumor suppressor gene commonly inactivated in a wide range of human cancers. However, initial genetic analysis of p18^{INK4c} status in >100 human tumor samples derived from >20 different tissues failed to identify homozygous deletions and found only a single sample with a putative somatic missense mutation (10).

Detailed biochemical studies provided further information on the role of p18^{INK4c} in inhibition of cdks. Initial studies suggested that p18^{INK4c} preferentially inhibited cdk6-containing complexes whereas p16^{INK4a} preferentially inhibited cdk4-containing complexes (8). This suggested that despite their status as homologues, these proteins have evolved for the purpose of providing subtly distinct negative regulation of cdks in the human cell cycle. However, this notion that INK4 proteins have distinct binding specificities for cdk4 and cdk6 remains somewhat controversial (11).

Mouse Models of p18^{INK4c} Deficiency Show Its Role as a Potent Tumor Suppressor

In contrast to the early data suggesting that p18^{INK4c} might play a limited role in human cancer pathogenesis, data from knockout mouse studies have shown a clear role for p18^{INK4c} in cellular proliferation, organ size, and cancer. p18^{INK4c}-deficient mice are viable but show a variety of intriguing phenotypes. Franklin and colleagues (12) reported that p18^{INK4c}-deficient mice displayed pronounced gigantism, organomegaly, lymphoma, and pituitary hyperplasia, phenotypes that were strikingly reminiscent of those seen in Rb^{-/-} mice. Shortly thereafter, Latres and colleagues (13) reported that p18^{INK4c}-deficient mice also displayed deregulated epithelial cell growth in the kidneys and breast. Interestingly, Bai and colleagues reported that p18^{INK4c} heterozygous (+/-) knockout

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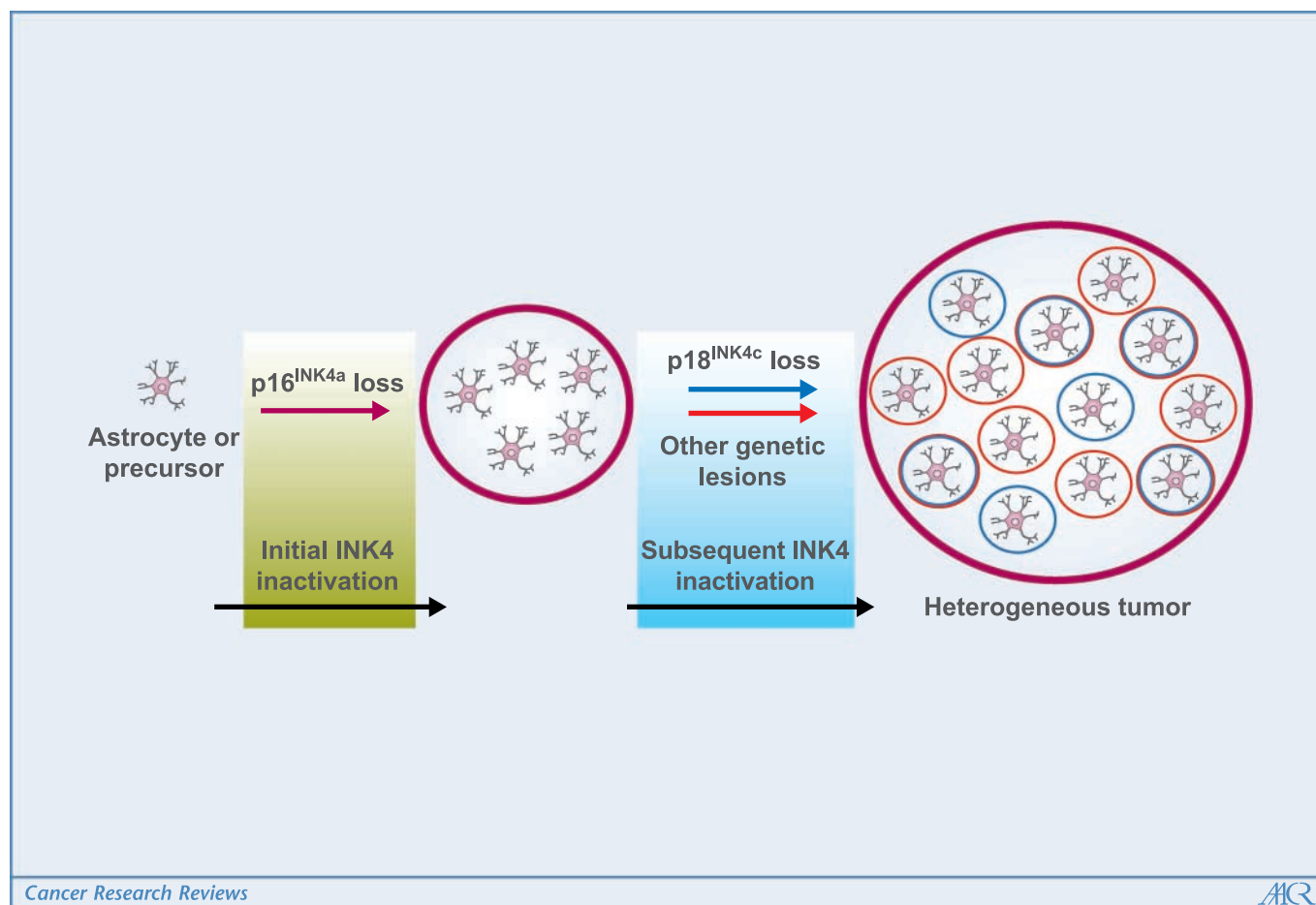


Figure 1. Depiction of sequential INK4 inactivation during GBM progression resulting in a tumor with homogeneous loss of p16^{INK4a} and heterogeneous loss of p18^{INK4c}.

mice developed tumors at an accelerated rate when treated with a chemical carcinogen. These tumors retained the remaining allele of p18^{INK4c}, showing that p18^{INK4c} is a haploinsufficient tumor suppressor in mice (14).

Subsequent studies in which p18^{INK4c}-deficient mice were crossed with mice harboring targeted deletions of other tumor suppressor genes uncovered additional roles for p18^{INK4c} in cancer pathogenesis. A subset of these studies are described here. Mice lacking both p18^{INK4c} and p53 were predisposed to medulloblastomas, hemangiosarcomas, and other tumors not present in either of the parental strains (15). Deletion of p18^{INK4c} in Ptc1^{+/-} mice led to the rapid development of medulloblastoma with high penetrance, and further examination of these and related mice indicated that p18^{INK4c} plays an important role in normal cerebellar development (16–18). Deletion of p18^{INK4c} in PTEN^{+/-} mice led to an enhanced predisposition to a variety of tumors when compared with mice with deletion of either gene alone, suggesting that these genes could synergize during the development of human malignancies harboring inactivation of both genes (such as GBM; ref. 19). Finally, mice lacking both p18^{INK4c} and p16^{INK4a} developed unusually aggressive pituitary tumors, suggesting that these GBM-related genes also synergize in tumor formation and that these INK4 family members could at least partially compensate for each other (20).

Evidence that p18^{INK4c} Is a Tumor Suppressor Gene in Human Cancer

Despite the early negative results in human cancer samples, the potential promise of p18^{INK4c} as a human tumor suppressor gene and the mouse studies implicating p18^{INK4c} as a bona fide tumor suppressor gene prompted several groups to continue to search for homozygous deletions and inactivating point mutations in a variety of common human cancer types. These efforts continued to prove largely frustrating, in that deletions/point mutations of p18^{INK4c} were identified rarely, albeit in a range of human tumor types including adenocarcinoma of the breast, multiple myeloma, acute lymphoblastic leukemia, oligodendroglioma, and meningioma (10, 21–30). Epigenetic inactivation of p18^{INK4c} in human medulloblastoma has also been reported (16).

Of these tumor sample types, cell lines derived from multiple myeloma harbored by far the highest frequency of p18^{INK4c} deletions, ~30%. Two studies in particular highlighted the frequency and effect of p18^{INK4c} deletion in multiple myeloma. In 2002, Kulkarni and colleagues (23) identified homozygous deletions of p18^{INK4c} in 6 of 16 multiple myeloma cell lines and showed that these deletions often occurred in tumor cells also harboring deletions of the p16^{INK4a}/p14^{ARF}/p15^{INK4b} locus. In 2006, Dib and colleagues (24) confirmed and extended this work, identifying

homozygous deletions of p18^{INK4c} in 13 of 40 multiple myeloma cell lines, but in only 2% to 10% of primary tumor samples. There were two opposing possible explanations for these data: the worrisome possibility that the majority of these p18^{INK4c} deletions in multiple myeloma cell lines could be artifacts of *ex vivo* culture, and the exciting possibility that deletions of p18^{INK4c} could be present in a similarly high fraction of primary tumor samples as well but that their presence was masked by intratumoral heterogeneity. These same issues would reemerge in the more recent studies implicating p18^{INK4c} in the pathogenesis of GBM.

Identification of Co-deletion of p18^{INK4c} and p16^{INK4a}/p14^{ARF}/p15^{INK4b} in Human GBM

In April 2008, Solomon and colleagues (6) and Wiedemeyer and colleagues (7) reported homozygous deletions of p18^{INK4c} in GBM. These deletions were identified using copy number analysis in GBM cell lines, xenografts, and primary tumors. Notably, Wiedemeyer and colleagues also identified three missense mutations of p18^{INK4c} that interfered with binding to cdk6. There were two similarities between the data presented in these studies and those previously reported for multiple myeloma. First, whereas p18^{INK4c} deletions were identifiable in GBM primary tumor samples, they were most commonly found in GBM cell lines and xenografts. Second, as in multiple myeloma, GBM samples with deletions of p18^{INK4c} often also harbored deletions of the p16^{INK4a}/p14^{ARF}/p15^{INK4b} locus. Follow-up functional studies clearly showed that p18^{INK4c} could play a tumor suppressor role in human and murine GBM, and Wiedemeyer and colleagues suggested that p18^{INK4c} could functionally compensate for p16^{INK4a} homozygous deletions during the pathogenesis of GBM.

Why the Disparity in p18^{INK4c} Deletions between Cultured and Uncultured GBMs?

One clear result of the GBM and multiple myeloma studies is that deletions of p18^{INK4c} are much easier to identify in cultured samples than in uncultured samples. There are at least two possible explanations for this disparity.

One possibility is that deletions of p18^{INK4c} are more easily identifiable in cultured samples because *ex vivo* growth enriches for p18^{INK4c}-deficient cells already present in the tumor. In fact, Solomon and colleagues showed just this: Whereas one primary GBM tumor they studied clearly harbored a deletion of p18^{INK4c}, identification of the deletion was obscured by intratumoral heterogeneity. However, that same deletion was easily identifiable in a first-passage xenograft derived from that tumor (6). That same primary tumor was homogeneous for deletion of the p16^{INK4a}/p14^{ARF}/p15^{INK4b} locus, providing support for the notion that genetic heterogeneity, and not the presence of admixed nonneoplastic cells, obscured detection of the p18^{INK4c} deletion in the primary tumor. Of note, it seems unlikely that the genetic heterogeneity displayed by these and other heterogeneous deletions was due to true haploinsufficiency because all p18^{INK4c} deletions identified by Solomon and colleagues and Wiedemeyer and colleagues in genetically homogeneous cell lines seemed to be biallelic/homozygous. Based on these types of data and immunohistochemistry studies identifying loss of p18^{INK4c} expression in ~40% of GBMs, Solomon and colleagues suggested that deletions of p18^{INK4c} could be common in GBM primary tumors but that intratumoral heterogeneity might obscure their easy identification.

However, it is also a formal possibility that the deletions of p18^{INK4c} identified by Solomon and colleagues and Wiedemeyer and colleagues in cultured GBM samples could have occurred *de novo* during *ex vivo* culture, and as such represent true culture artifacts. Importantly, however, both groups clearly identified deletions of p18^{INK4c} in both cultured and uncultured samples, providing strong evidence that deletions of p18^{INK4c} are not artifacts of culture. Furthermore, it is arguable whether any putative cancer-related gene has been shown to be deleted/mutated only in samples grown in an *ex vivo* setting, and some believe that the enthusiasm for the idea that *ex vivo* culture leads to artifactual genetic lesions exceeds the data to support it.

Homogeneity of p16^{INK4a}/p14^{ARF}/p15^{INK4b} Deletions in GBM

A related point brought up by Solomon and colleagues is that unlike the heterogeneous deletions of p18^{INK4c}, GBMs are remarkably homogeneous with regard to deletions of the p16^{INK4a}/p14^{ARF}/p15^{INK4b} locus. Emerging copy number analyses of GBM samples make it obvious that deletion of this locus is by far the most common homozygous deletion present in GBM (in >75% of samples), and that genetically heterogeneous uncultured primary GBMs tend to be homogeneous for deletion of this locus. This degree of homogeneity suggests that deletion of the p16^{INK4a}/p14^{ARF}/p15^{INK4b} locus is unusually fundamental to the pathogenesis of GBM, and, when taken together with the heterogeneity of deletions in p18^{INK4c}, suggests that deletion of the p16^{INK4a}/p14^{ARF}/p15^{INK4b} locus is an early event in the pathogenesis of GBM, whereas deletion of p18^{INK4c} is likely to be a later event associated with progression of GBM (Fig. 1). These data further suggest that deletion of the p16^{INK4a}/p14^{ARF}/p15^{INK4b} locus could even be the initiating event in GBM, as this would simultaneously explain the remarkable homogeneity of these deletions and the shockingly explosive growth of primary GBM.

Possible Explanations for Co-deletion of the p18^{INK4c} Gene and the p16^{INK4a}/p14^{ARF}/p15^{INK4b} Locus in GBM

One intriguing observation is that deletions of p18^{INK4c} generally coexist in the same GBM cells also harboring deletions of the p16^{INK4a}/p14^{ARF}/p15^{INK4b} locus. There are several possible explanations for this surprising finding. Wiedemeyer and colleagues showed that depletion of p16^{INK4a} led to transcriptional up-regulation of p18^{INK4c} by the E2F1 transcription factor, suggesting that p18^{INK4c} is induced to compensate for deletion of p16^{INK4a}, and that this provides selective pressure for the loss of p18^{INK4c} (7, 31). Such a model might be considered a "serial model," in that there is a linear mechanistic relationship between the tumor suppression activities of these two genes. However, it is also worth noting that (a) deletions of p16^{INK4a} are at least twice as common in GBM than deletions of p18^{INK4c}; (b) GBMs are quite heterogeneous for deletions of p18^{INK4c} yet remarkably homogeneous for deletions of the p16^{INK4a}/p14^{ARF}/p15^{INK4b} locus; and (c) the cdk inhibitors encoded by these loci probably have distinct specificities for binding to different cdks. These observations suggest that INK4 genes could play biologically distinct roles in the initiation (p16^{INK4a}) and progression (p18^{INK4c}) of GBM. Such a model might be termed a "parallel model," in that inactivation of

these genes is mechanistically unrelated and loss of each gene has distinct biochemical and phenotypic sequelae during the pathogenesis of GBM.

Conclusions and Future Directions

Numerous studies have now implicated p18^{INK4c} as a tumor suppressor whose genetic inactivation plays a role in the pathogenesis of GBM, multiple myeloma, and a variety of other common cancers. Deletions of p18^{INK4c} are clearly less common than deletions of the p16^{INK4a}/p14^{ARF}/p15^{INK4b} locus and seem to show substantial intratumoral heterogeneity. They often occur in tumors also harboring deletions of the p16^{INK4a}/p14^{ARF}/p15^{INK4b}

locus but probably occur later in tumorigenesis, raising fundamental questions about the relative contributions of these two loci to the initiation and progression of human neoplasia. Further studies are clearly warranted to better define the relative biochemical and phenotypic roles of these INK4 family members in the pathogenesis of GBM and other human tumor types.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- Furnari FB, Fenton T, Bachoo RM, et al. Malignant astrocytic glioma: genetics, biology, and pathways to treatment. *Genes Dev* 2007;21:2683–710.
- Jen J, Harper JW, Bigner SH, et al. Deletion of p16 and p15 genes in brain tumors. *Cancer Res* 1994;54:6353–8.
- Schmidt EE, Ichimura K, Reifenger G, Collins VP. CDKN2 (p16/MTS1) gene deletion or cdk4 amplification occurs in the majority of glioblastomas. *Cancer Res* 1994;54:6321–4.
- Ruas M, Peters G. The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta* 1998;1378:F115–77.
- Lowe SW, Sherr CJ. Tumor suppression by Ink4a-Arf: progress and puzzles. *Curr Opin Genet Dev* 2003;13:77–83.
- Solomon DA, Kim JS, Jenkins S, et al. Identification of p18^{INK4c} as a tumor suppressor gene in glioblastoma multiforme. *Cancer Res* 2008;68:2564–9.
- Wiedemeyer R, Brennan C, Haffner TP, et al. Feedback circuit among INK4 tumor suppressors constrains human glioblastoma development. *Cancer Cell* 2008;13:355–64.
- Guan KL, Jenkins CW, Li Y, et al. Growth suppression by p18, a p16^{INK4/MTS1}- and p14^{INK4B/MTS2}-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes Dev* 1994;8:2939–52.
- Hirai H, Roussel MF, Kato JY, Ashmun RA, Sherr CJ. Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Mol Cell Biol* 1995;15:2672–81.
- Zariwala M, Liu E, Xiong Y. Mutational analysis of the p16 family cyclin-dependent kinase inhibitors p15INK4b and p18INK4c in tumor-derived cell lines and primary tumors. *Oncogene* 1996;12:451–5.
- Thullberg M, Bartkova J, Khan S, et al. Distinct versus redundant properties among members of the INK4 family of cyclin-dependent kinase inhibitors. *FEBS Lett* 2000;470:161–6.
- Franklin DS, Godfrey VL, Lee H, et al. CDK inhibitors p18(INK4c) and p27(Kip1) mediate two separate pathways to collaboratively suppress pituitary tumorigenesis. *Genes Dev* 1998;12:2899–911.
- Latres E, Malumbres M, Sotillo R, et al. Limited overlapping roles of p15(INK4b) and p18(INK4c) cell cycle inhibitors in proliferation and tumorigenesis. *EMBO J* 2000;19:3496–506.
- Bai F, Pei XH, Godfrey VL, Xiong Y. Haploinsufficiency of p18(INK4c) sensitizes mice to carcinogen-induced tumorigenesis. *Mol Cell Biol* 2003;23:1269–77.
- Zindy F, Nilsson LM, Nguyen L, et al. Hemangiosarcomas, medulloblastomas, and other tumors in Ink4c/p53-null mice. *Cancer Res* 2003;63:5420–7.
- Uziel T, Zindy F, Xie S, et al. The tumor suppressors Ink4c and p53 collaborate independently with Patched to suppress medulloblastoma formation. *Genes Dev* 2005;19:2656–67.
- Zindy F, Knoepfler PS, Xie S, Sherr CJ, Eisenman RN, Roussel MF. N-Myc and the cyclin-dependent kinase inhibitors p18INK4c and p27Kip1 coordinately regulate cerebellar development. *Proc Natl Acad Sci U S A* 2006;103:11579–83.
- Cunningham JJ, Roussel MF. Cyclin-dependent kinase inhibitors in the development of the central nervous system. *Cell Growth Differ* 2001;12:387–96.
- Bai F, Pei XH, Pandolfi PP, Xiong Y. p18INK4c and Pten constrain a positive regulatory loop between cell growth and cell cycle control. *Mol Cell Biol* 2006;26:4564–76.
- Ramsey MR, Krishnamurthy J, Pei XH, et al. Expression of p16INK4a compensates for p18INK4c loss in cyclin-dependent kinase 4/6-dependent tumors and tissues. *Cancer Res* 2007;67:4732–41.
- Lapointe J, Lachance Y, Labrie Y, Labrie C. A p18 mutant defective in CDK6 binding in human breast cancer cells. *Cancer Res* 1996;56:4586–9.
- Tasaka T, Berenson J, Vescio R, et al. Analysis of the p16INK4A, p15INK4B and p18INK4C genes in multiple myeloma. *Br J Haematol* 1997;96:98–102.
- Kulkarni MS, Daggett JL, Bender TP, Kuehl WM, Bergsagel PL, Williams ME. Frequent inactivation of the cyclin-dependent kinase inhibitor p18 by homozygous deletion in multiple myeloma cell lines: ectopic p18 expression inhibits growth and induces apoptosis. *Leukemia* 2002;16:127–34.
- Dib A, Peterson TR, Raducha-Grace L, et al. Paradoxical expression of INK4c in proliferative multiple myeloma tumors: bi-allelic deletion vs increased expression. *Cell Div* 2006;1:23.
- Iolascon A, Faienza MF, Coppola B, della Ragione F, Schettini F, Biondi A. Homozygous deletions of cyclin-dependent kinase inhibitor genes, p16(INK4A) and p18, in childhood T cell lineage acute lymphoblastic leukemias. *Leukemia* 1996;10:255–60.
- Pohl U, Cairncross JG, Louis DN. Homozygous deletions of the CDKN2C/p18INK4C gene on the short arm of chromosome 1 in anaplastic oligodendrogliomas. *Brain Pathol* 1999;9:639–43.
- Husemann K, Wolter M, Buschges R, Bostrom J, Sabel M, Reifenger G. Identification of two distinct deleted regions on the short arm of chromosome 1 and rare mutation of the CDKN2C gene from 1p32 in oligodendroglial tumors. *J Neuropathol Exp Neurol* 1999;58:1041–50.
- He J, Hoang-Xuan K, Marie Y, et al. p18 tumor suppressor gene and progression of oligodendrogliomas to anaplasia. *Neurology* 2000;55:867–9.
- Santarius T, Kirsch M, Nikas DC, Imitola J, Black PM. Molecular analysis of alterations of the p18INK4c gene in human meningiomas. *Neuropathol Appl Neurobiol* 2000;26:67–75.
- Bostrom J, Meyer-Puttitz B, Wolter M, et al. Alterations of the tumor suppressor genes CDKN2A (p16(INK4a)), p14(ARF), CDKN2B (p15(INK4b)), and CDKN2C (p18(INK4c)) in atypical and anaplastic meningiomas. *Am J Pathol* 2001;159:661–9.
- Blais A, Monte D, Pouliot F, Labrie C. Regulation of the human cyclin-dependent kinase inhibitor p18INK4c by the transcription factors E2F1 and Sp1. *J Biol Chem* 2002;277:31679–93.